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Abstract: Aims Dysregulation of the bone morphogenetic protein receptor type 2 (BMPR2) is a hallmark feature that has been described in several forms of pulmonary hypertension. We recently identified the microRNA miR-20a within a highly conserved pathway as a regulator of the expression of BMPR2. To address the pathophysiological relevance of this pathway in vivo, we employed antagomiR-20a and investigated whether specific inhibition of miR-20a could restore functional levels of BMPR2 and, in turn, might prevent pulmonary arterial vascular remodelling. Methods and results For specific inhibition of miR-20a, cholesterol-modified RNA oligonucleotides (antagomiR-20a) were synthesized. The experiments in mice were performed by using the hypoxia-induced mouse model for pulmonary hypertension and animal tissues were analysed for right ventricular hypertrophy and pulmonary arterial vascular remodelling. Treatment with antagomiR-20a enhanced the expression levels of BMPR2 in lung tissues; moreover, antagomiR-20a significantly reduced wall thickness and luminal occlusion of small pulmonary arteries and reduced right ventricular hypertrophy. To assess BMPR2 signalling and proliferation, we performed in vitro experiments with human pulmonary arterial smooth muscle cells (HPASMCs). Transfection of HPASMCs with antagomiR-20a resulted in activation of downstream targets of BMPR2 showing increased activation of Id-1 and Id-2. Proliferation of HPASMCs was found to be reduced upon transfection with antagomiR-20a. Conclusion This is the first report showing that miR-20a can be specifically targeted in an in vivo model for pulmonary hypertension. Our data emphasize that treatment with antagomiR-20a restores functional levels of BMPR2 in pulmonary arteries and prevents the development of vascular remodelling.

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AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension

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Abstract

Aims. Dysregulation of the bone morphogenetic protein receptor type 2 (BMPR2) is a hallmark feature that has been described in several forms of pulmonary hypertension. We recently identified the microRNA miR-20a within a highly conserved pathway as regulator of the expression of BMPR2. To address the pathophysiological relevance of this pathway *in vivo*, we employed antagomiR-20a and investigated whether specific inhibition of miR20a could restore functional levels of BMPR2 and, in turn, might prevent pulmonary arterial vascular remodelling.

Methods and results. For specific inhibition of miR-20a, cholesterol modified RNA oligonucleotides (antagomiR-20a) were synthesized. The experiments in mice were performed by using the hypoxia-induced mouse model for pulmonary hypertension and animal tissues were analysed for right ventricular hypertrophy and pulmonary arterial vascular remodelling. Treatment with antagomiR-20a enhanced the expression levels of BMPR2 in lung tissues; moreover, antagomiR-20a significantly reduced wall thickness and luminal occlusion of small pulmonary arteries and reduced right ventricular hypertrophy. To assess BMPR2 signalling and proliferation, we performed *in vitro* experiments with human pulmonary arterial smooth muscle cells (HPASMC). Transfection of HPASMC with antagomiR-20a resulted in activation of downstream targets of BMPR2 showing increased activation of Id-1 and -Id2. Proliferation of HPASMC was found to be reduced upon transfection with antagomiR-20a.

Conclusion. This is the first report showing that miR-20a can be specifically targeted in an *in vivo* model for pulmonary hypertension. Our data emphasize that treatment with antagomiR-20a restores functional levels of BMPR2 in pulmonary arteries and prevents the development of vascular remodelling.

Keywords

BMPR2, pulmonary hypertension, microRNA, antagomiR, vascular remodelling

Introduction

Vascular remodelling of small pulmonary arteries is, together with vasoconstriction and microthrombosis, one of the pathogenetic hallmarks of pulmonary arterial hypertension (PAH). However, it is largely unknown what causes vascular remodelling or how it can be treated. Genetic mutations of the bone morphogenetic protein receptor type II (BMPR2), a member of the transforming growth factor (TGF- β) family expressed on the surface of endothelial and vascular smooth muscle cells of the pulmonary arterial circulation, have been described in familial and idiopathic PAH^{1, 2}. Moreover, reduced expression of BMPR2 without concomitant mutations has also been found in other forms of pulmonary hypertension including established animal models of the disease^{3, 4, 5}. It has been debated controversially whether the altered expression of BMPR2 due to mutations or downregulation might have a functional readout and, thus, an impact on the pathogenesis of PAH. Two recent studies however have underscored the potential role of BMPR2-mediated signalling for vascular remodelling^{6, 7}. Long and coworkers observed that dysfunctional BMPR2/TGF- β signal transduction results in altered expression of transcription factors including Smads and Id (inhibitor of DNA binding) leading to migration of vascular smooth muscle cells⁶. Similar findings have been reported by another study showing that heterogeneous mutations of BMPR2 are associated with disrupted Smad signalling resulting in a pro-proliferative and anti-apoptotic phenotype of pulmonary arterial smooth muscle cells⁷.

MicroRNAs (miRNAs) comprise a class of small, non-coding RNA molecules that regulate gene expression on the post-transcriptional level⁸; miRNAs preferentially bind to conserved regions in the 3' untranslated region (UTR) of their target genes and either suppress the translation or the mRNA stability of these genes⁹. There is a growing body of evidence that the abnormal expression of miRNAs modulates human diseases by regulating key molecules of apoptosis, proliferation or cell signalling¹⁰. Conversely, efficient and non-toxic inhibition of miRNAs *in vivo* is of particular interest since such methods would offer a therapeutic approach to suppress altered miRNA expression in human disease. Krutzfeldt and co-workers recently described the inhibition of miRNAs by the

application of small anti-sense RNA molecules conjugated with cholesterol called antagomiRs in mice¹¹.

We have recently proposed that miRNAs might regulate the expression of BMPR2 and identified a phylogenetically conserved pathway involving the action of the signal transducer and activator of transcription (STAT)3 and the microRNA cluster miR-17/92, mainly of miR-20a¹². Others have emphasised this hypothesis by showing that miRNAs derived from miR- 17/92 are over expressed in experimental pulmonary hypertension *in vivo*¹³ making these miRNAs interesting targets for further investigations. With respect to pathogenesis and therapy, this model offers the potential to restore functional expression levels of BMPR2 by antagonising distinct miRNAs.

In the present study, we addressed feasibility and functional readout of specific antagonisation of miR20-a by using antagomiR in an animal model of hypoxia-induced pulmonary hypertension *in vivo* and *in vitro*.

Methods

Animal experiments

Male mice (BL6) were obtained by the Institute for Veterinary Physiology at the University of Zurich. A total of 40 animals was used, 8 of these were employed to adjust dosage and timing of the intraperitoneal (i.p.) applications of antagomiR-20a (pre-test). The study design of the *in vivo* experiments is provided in Figure 1a. Four groups were used (n= 8 each): 3 groups were employed in hypoxic conditions. The fraction of oxygen was decreased gradually from 21% to 10% over 60 minutes. Hypoxic conditions were provided by chambers connected to a gas mixer (Ruskin Life Science). Animals were treated with antagomiR-20a (25 mg/kg), antagomiR negative control (antagomiR_MM) or PBS, respectively. Injections were performed at baseline (day 0) with boosters performed at day 5 and 12. One group of animals was used as normoxic controls. Mice were assessed daily for activity and well-being. Food and water was provided ad libitum. Blood analysis, morphometry and tissue preparation for later analysis were performed on day 21. The experiments were approved by Zurich Canton's Veterinary office. To assess morphometry, hearts and lungs were removed, formalin-fixed without prior perfusion, paraffin-embedded and stained with hematoxylin and eosin. Parameters including heart hypertrophy, cell wall thickness, and vessel occlusion were determined by using the MCID analysis program 7.0 (InterFocus Imaging, Cambridge, UK).

AntagomiR design

Cholesterol modified RNA oligonucleotides (antagomiRs) directed against human and murine miR-20a (MIMAT0000529) were designed as described ⁽¹¹⁾ and synthesized by Microsynth (Microsynth, Balgach, Switzerland). As a negative control, twelve point mutations were introduced into the miR-20a mature sequence (antagomiR_MM) creating a RNA sequence which is not encoded in the murine or human genome. The sequences are the following: antagomiR-20a (5' – CUA CCU GCA CUA UAA GCA CUU UA – 3'), antagomiR_MM (5' – GAC CGU UCA CUA UUA CGA GUC AA – 3').

Statistics

For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. To compare samples with parametric distribution, the paired or unpaired t-test was applied and a p-value < 0.05 was considered to be statistically significant (* p < 0.05, ** p < 0.01, *** p < 0.001). For statistical analysis between more than two groups one-way ANOVA (Tukey post-test) was used. In all statistical analyses, two sided tests were applied. A table showing all statistical analyses is provided (Suppl. table 1). All data are shown as mean \pm SD.

Extensive materials and methods are provided in the online supplement.

Results

Application of antagomiR-20a suppresses miR-20a expression in target tissues

We recently identified a novel signalling pathway that regulates the expression of BMPR2 through the action of miR-20a¹². Here we addressed the implications of this pathway in an animal model of hypoxia-induced pulmonary hypertension. The *in vivo* experiments were performed as described in the Methods (Fig. 1a). As shown in Figure 1b, repeated intraperitoneal injections of antagomiR-20a significantly down regulated the expression levels of miR-20a in lung tissues when assessed by qPCR at day 21. AntagomiR-20a treated animals showed an expression level of miR-20a of $\Delta\text{Ct } 3.68 \pm 0.58$ as compared to $\Delta\text{Ct } 2.31 \pm 0.23$ in normoxic control animals thus resulting in a reduction of 59.3% ($p < 0.001$). On the other hand, no significant changes were observed between normoxic control mice and antagomiR_mismatch (antagomiR_MM, $\Delta\text{Ct } 2.46 \pm 0.2$) or PBS ($\Delta\text{Ct } 2.18 \pm 0.26$) treated animals. Similar results were observed for other tissues analysed, in particular heart and liver (data not shown). These data indicate that intraperitoneally injected antagomiRs are not subjected to a significant hepatic first-pass effect and, thus, are a feasible approach to inhibit the expression of distinct miRNAs in target tissues including the lungs. The sequences of antagomiR-20a and antagomiR_MM are provided in Figure 1c.

AntagomiR-20a prevents the development of hypoxia-induced morphological changes within the cardiopulmonary circulation

Morphometric analyses were performed to assess the functional and hemodynamic consequences of reduced expression levels of miR-20a achieved by the application of antagomiR-20a. Representative images of pulmonary arterioles stained for α -smooth muscle actin are shown in Figure 2a; treatment with antagomiR-20a significantly inhibited the vascular remodelling observed in PBS and antagomiR_MM treated hypoxic animals. Computer-based analysis further quantified the occlusion of these vessels to be reduced from 13.47 ± 0.73 and 13.33 ± 0.61 (antagomiR_MM and PBS treated animals, respectively) to 7.72 ± 0.93 in antagomiR-20a treated mice or to 1.73 ± 0.48 in normoxic controls (Fig. 2b). Similar effects were observed for the thickness of the vessel wall showing a

reduction from $1.18 \pm 0.06 \mu\text{M}$ and $1.28 \pm 0.15 \mu\text{M}$ in antagomiR_MM and PBS treated animals to $0.96 \pm 0.05 \mu\text{M}$ in antagomiR-20a treated animals ($0.71 \pm 0.04 \mu\text{M}$ in normoxic controls; Fig. 2c).

Right ventricular hypertrophy, assessed in Figure 2d, after 10% O₂ for 21 days was strongly reduced in animals receiving antagomiR-20a when compared to hypoxic mice treated with antagomiR_MM or PBS (representative images are shown in Fig. 2d). These results were confirmed by calculating heart weight relative to body weight (antagomiR_MM: 0.0061 ± 0.0006 vs. antagomiR-20a: 0.0056 ± 0.0007 ; Fig. 2e) and by the relation of the right ventricular to the left ventricular volume (RV/LV + S; antagomiR_MM: 0.25 ± 0.01 ; antagomiR-20a: 0.21 ± 0.01 ; Fig. 2f), indicating that the right ventricular afterload was reduced in antagomiR-20a treated mice as compared to mock-treated hypoxic animals. Blood analysis performed at day 21 revealed that hypoxia resulted in erythrocytosis, which was most prominent in hypoxic PBS-treated animals (Fig. 2g). Whereas significant differences were observed between normoxic controls ($11.63 \pm 0.91 \text{ g/dl}$) and all hypoxic groups ($20.23 \pm 2.04 \text{ g/dl}$ for antagomiR-20a, $21.75 \pm 1.28 \text{ g/dl}$ for antagomiR_MM, and $23.27 \pm 1.31 \text{ g/dl}$ for PBS, respectively), differences between antagomiR-20a and antagomiR_MM treated animals did not reach statistical significance.

These data imply that the pathomorphological sequelae of hypoxia on the pulmonary vasculature, which lead to the development of pulmonary hypertension and, subsequently, to right ventricular hypertrophy, can be prevented by the application of antagomiR-20a.

AntagomiR-20a restores functional expression levels of BMPR2 in mice

Since we hypothesized that the reduced expression levels of BMPR2 observed in pulmonary hypertension might be due to targeted inhibition by miR-20a¹², we next investigated whether the application of specific antagomiRs to mice increase BMPR2 expression levels, and whether restoration of this receptor might have functional implications. As shown in Figure 3a, the mRNA expression levels of BMPR2 in lung tissue were found to be reduced in both hypoxic control groups (antagomiR_MM: $\Delta\text{Ct } 3.36 \pm 0.73$; PBS: $\Delta\text{Ct } 3.2 \pm 0.41$) when compared to normoxic mice ($\Delta\text{Ct } 2.61 \pm 0.19$). Interestingly, mRNA levels of BMPR2 under hypoxic conditions could be normalised to the

levels observed in normoxic control animals by the application of antagomiR-20a ($\Delta\text{Ct } 2.68 \pm 0.16$, Fig. 3a). These findings are further illustrated by immunohistochemical and Western blot analysis using antibodies directed against BMPR2 showing that the application of antagomiR-20a increased the expression of BMPR2 on endothelial and smooth muscle cells of pulmonary arterioles as compared to antagomiR_MM injected mice (Fig. 3b) and PBS treated hypoxic mice (Fig. 3c, Western blots provided as supplementary Fig. S1).

Similarly, Smad5 and Id-2, two validated signalling elements downstream of the BMPR2 pathway¹⁴ showed enhanced expression levels in lung tissues of animals treated with antagomiR-20a as compared to hypoxic controls (Smad5: antagomiR-20a $\Delta\text{Ct } 3.89 \pm 0.2$ vs. hypoxia PBS $\Delta\text{Ct } 4.16 \pm 0.26$, Fig. 3d; Id-2: antagomiR-20a $\Delta\text{Ct } 5.09 \pm 0.46$ vs. hypoxia PBS $\Delta\text{Ct } 5.68 \pm 0.52$, Fig. 3e). Expression levels for Id-1, however, were not found to be changed in normoxic or hypoxic animals (Supplementary Fig. S2). Protein analysis by Western blotting for Id-2 further confirmed these findings and are provided as supplementary information (Supplementary Fig. S3). These data suggest that treatment with antagomiR-20a increases expression levels of BMPR2 within the pulmonary vasculature and, most important, restored functional BMPR2 signalling.

AntagomiR-20a enhances BMP-2 mediated signalling *in vitro*

Upon binding of the ligand BMP-2 to BMPR2, Smad5 is activated by phosphorylation, which, in turn, activates the transcription factors Id-1 and -2. Since treatment with antagomiR-20a up regulated the expression levels of BMPR2 *in vivo*, we next investigated whether antagonisation of miR-20a might also enhance the BMP-2 mediated signalling *in vitro*. We thus utilised human pulmonary arterial smooth muscle cells (HPASMC) that were stimulated with BMP-2 for different time periods and found that treatment with antagomiR-20a enhanced BMPR2-induced signalling activity, i.e. the expression levels of Id-1 and -2 showed an augmentation of the BMP-2 mediated induction of gene expression by transfection of antagomiR-20a. As shown in Fig. 4a, 1h of BMP-2 stimulation enhanced the mRNA levels of Id-1 in antagomiR-20a treated cells by 2.65 ± 1.55 fold (as compared to 1.52 ± 0.5 fold in antagomiR_MM transfected cells, $p = 0.102$). After 4h of BMP-2 stimulation Id-1 was significantly more induced in HPASMC treated with antagomiR-20a (4 ± 2.28) as compared to control

cells (3 ± 2.14 , $p = 0.035$). The mRNA levels of Id-2, on the other hand, were found to be significantly more elevated by BMP-2 stimulation in antagomiR-20a treated HPASMC after 1h only (antagomiR_MM: 1.14 ± 0.31 fold, antagomiR-20a: 1.65 ± 0.41 fold, $p = 0.02$, Fig. 4b).

Finally, promoter studies were performed to illustrate an enhancement of BMP-2 signalling by antagonising miR-20a. The promoter of Id-1 containing multiple Smad transcription factor binding sites¹⁵ was cloned into a luciferase-based reporter gene vector. As shown in Fig. 4d, 4h of BMP-2 stimulation induced the relative promoter activity of Id-1 by 1.38 ± 0.22 fold in antagomiR_MM transfected cells. Interestingly, the increase in promoter activity upon stimulation with BMP-2 was significantly more enhanced when cells were transfected with antagomiR-20a (1.69 ± 0.22 fold, $p=0.016$). Conversely, the over expression of miR-20a by transfection of small RNA oligonucleotides (mat-miR-20a) reduced the BMP-2 mediated induction of the promoter activity of Id-1 (mat-miR_MM: 1.81 ± 0.27 fold; mat-miR-20a: 1.47 ± 0.35 fold, $p = 0.013$; Fig. 4e).

Note that, Smad5 was predicted to be directly targeted by miR-20a (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org,¹⁶). Accordingly, the expression of Smad5 after silencing of miR-20a in HPASMC was analysed. As shown in supplementary Fig. S4, transfection of antagomiR-20a significantly increased mRNA levels of Smad5 after 72h, but failed to enhance protein levels. Moreover, a reporter gene assay comprising the predicted seed match of miR-20a in the 3' untranslated region (UTR) of Smad5 could not show a direct miRNA - mRNA interaction. Phosphorylation of Smad5 (normalised to Smad5 protein levels), however, showed a trend towards enhanced phosphorylation activity in stimulated as well as in unstimulated conditions as compared to antagomiR_MM controls, but these data did not reach statistical significance (supplementary Fig. S5).

Proliferation of vascular smooth muscle cells is reduced upon transfection with antagomiR-20a

Proliferation of pulmonary arterial smooth muscle cells is an essential part of the vascular remodelling in pulmonary hypertension; we thus addressed whether transfection with antagomiR-20a affects proliferation assessed by the BrdU assay. As shown in Figure 5a, proliferation was significantly

reduced in antagomiR-20a transfected HPASMC (A_{450nm} : from 1.229 ± 0.182 to 0.867 ± 0.3) resulting in a reduction by 29.5% as compared to antagomiR_MM transfected controls ($p = 0.018$); conversely, when cells were transfected with precursor molecules of miR-20a (pre-miR-20a), proliferation was significantly increased (A_{450nm} : from 1.049 ± 0.223 to 1.576 ± 0.418 , or by 50.2 %, respectively. $p = 0.012$). On the other hand, no significant changes were seen when apoptosis was analysed (Supplementary Fig. S6). AntagomiR-20a transfection of HPASMC resulted after 24h in up regulation of the cell cycle inhibitor p21 on protein level (antagomiR_MM: 0.232 ± 0.112 vs. antagomiR-20a: 0.804 ± 0.077 , $p < 0.001$ Fig. 5b) as well as on mRNA level (1.53 ± 0.43 fold, $p = 0.051$ Fig. 5c). The expression of miR-20a was analysed in the same samples showing down regulation of miR-20a in HPASMC by antagomir-20a treatment (Fig. 5d).

Discussion

We have recently identified a microRNA-mediated signalling pathway that regulates the expression of BMPR2 on human pulmonary arterial endothelial cells ¹². While this pathway might explain, at least *in vitro*, the reduced expression of BMPR2 as observed in several forms of pulmonary hypertension, the functional relevance of this pathway was unclear so far. A very recent study has confirmed the pathogenetic importance of the microRNA cluster 17/92 by inhibition of miR-17 in experimental pulmonary hypertension ¹⁷; based on our previous work that identified two functionally related miRs as regulators of BMPR2 expression, i.e. miR-17 and miR-20a but the latter one being more potent ¹², we focused here on the action of miR-20a.

In the present study we employed an established animal model of pulmonary hypertension and found that i) intraperitoneal injection of antagomiR in general is a feasible approach to efficiently downregulate distinct miRNAs in target tissues; ii) antagonisation of miR-20a reduces the hypoxia-induced remodelling of pulmonary arterioles and, subsequently, reduces right heart hypertrophy in hypoxic animals; and, iii), that treatment with antagomiR-20a restores functional expression levels of BMPR2 both *in vivo* and *in vitro*.

In these experiments, antagonisation of miR-20a significantly reduced the vascular remodelling in hypoxic animals as assessed by wall thickness and luminal occlusion of small pulmonary arteries. Thickness of the vessel wall was less prominent than in hypoxic control animals, probably due to a lesser grade of muscularization upon reduced proliferation of vascular smooth muscle cells in the medial layer. The vascular occlusion of these vessels that is commonly observed in pulmonary hypertension and might result from vasoconstriction, proliferation of the intimal and medial layer and the development of plexiform lesions, has also been found to be reduced significantly in antagomiR-20a treated animals. Vascular occlusion and narrowing of the pulmonary arterial lumen increase pulmonary vascular resistance and right ventricular afterload; these alterations result in cardiac hypertrophy and, ultimately, right heart failure. In our experiments, antagomiR-20a treated animals revealed significantly less right ventricular hypertrophy than their mock-treated hypoxic controls, indicating improved pulmonary arterial hemodynamics in antagomiR-20a treated mice. However,

since the expression of miR-20a has been antagonised not only in the lungs, it remains unclear whether the observed reduction of right ventricular hypertrophy is an indirect effect of reduced pulmonary arterial pressure or, alternatively, whether it might be due to concomitant inhibition of miR-20a in cardiac myocytes. In contrast to other miRNAs, including miR-133¹⁸, miR-21¹⁹ or miR-27b²⁰ that also have been antagonised experimentally, miR-20a has not been described to be overexpressed in response to cardiac stress. With regard to other cell types, treatment with antagomiR-20a did not significantly alter the hypoxia-induced polycythemia of erythrocytes, indicating that, at least, no such pleiotropic effects by antagonising miR-20a have occurred on hematopoietic cells.

We further showed that treatment with antagomiR-20a increased the expression levels of BMPR2 in lung tissue, thus confirming indirectly that miR-20a targets the mRNA of BMPR2 as it was suggested previously by our *in vitro* data on human pulmonary arterial endothelial cells. Here, transfection of cells with antagomiR-20a could rescue expression and downstream signalling events of BMPR2 that was silenced by siRNA (supplemental material, Fig. S6). It has been discussed controversially, whether the observed dysregulation of BMPR2 in several forms of pulmonary hypertension might be of pathogenetic relevance. However, genetic studies and recent *in vitro* data suggest that BMPR2 and its downstream signalling play an important role in cell proliferation, vascular remodelling and, thus, in the development of pulmonary hypertension. For instance, Wong and co-workers showed that the activation of the BMPR2 signalling pathway in HPASMC leads to the inhibition of proliferation probably due to the induction of the cell cycle repressor p21²¹. Our findings are along the line of these data and further provide evidence both *in vivo* and *in vitro* that a functional BMPR2 signalling prevents major vascular remodelling within small pulmonary arteries. In particular, we showed that the restoration of BMPR2 expression in lung tissue leads to activation of the BMP-2 target Id-2. Similarly, *in vitro*, stimulation of HPASMC with BMP-2 and transfection with antagomiR-20a revealed increased BMP-2 signalling as indicated by enhanced promoter activity of Id-1 and up regulated expression levels of Id-1 and -2. These transcription factors have been associated with cell proliferation²²; activated Id-1/-2 thus would reduce the proliferation rate of cells. Here, levels of Id-1 and -2 have been found to be increased in antagomiR-20a treated HPASMC, and, in subsequent experiments, the proliferation of HPASMC was reduced by transfection of antagomiR-20a, whereas

apoptosis was not affected significantly. These data strongly indicate that the restoration of BMPR2 by treatment with antagomiR-20a is functional and, thus, reconstitutes the downstream signalling of BMPR2 in pulmonary arterial smooth muscle cells and in lung tissue. The reduced proliferation of smooth muscle cells was further shown to be associated with enhanced expression of the cell cycle repressor p21. We suggest that the observed increased expression of p21 by antagomiR-20a is caused by two ally acting mechanisms. On the one hand it was shown that miR-20a directly regulates the expression of p21²³, and on the other hand Wong et al. demonstrated that BMP-2 signalling enhances protein levels of p21²¹. Therefore, antagomiR-20a treatment increases p21 levels by the inhibition of miR-20a and by enhancing BMP-2 signalling, which might result in growth arrest of smooth muscle cells. In summary, functional restoration of BMPR2 appears to inhibit the development of hypoxia-induced vascular remodelling by enhanced intracellular signalling activity and reduced proliferation.

Finally, antagonisation of miR-20a, one of the key microRNAs in pulmonary hypertension, was successfully achieved in hypoxic mice by repeated intraperitoneal injections. Since antagomiRs are employed to a growing extent for antagonisation of distinct microRNAs for pathogenetic investigations and therapeutic purposes¹¹, these findings make antagomiRs to interesting tools for several reasons: downregulation of miR-20a was observed in target organs behind the liver, a finding that mutually excludes a significant hepatic first-pass effect; the effects have been longstanding, thus minimising the need for frequent applications; toxic effects have not been observed in terms of well-being of the animals or interferon-gamma expression in liver tissue (data not shown). Moreover, to our knowledge, this is the first report on intraperitoneal application of antagomiRs.

Our data might be limited by the fact that the increase of miR-20a observed under hypoxic conditions did not reach statistical significance. However, miRs are considered to act as fine tuners of gene regulation, and, thus, even small and non-significant elevations might have functional relevance. Accordingly, the increase of miR-20a and other members of the cluster miR-17/92 was rather small in other *in vivo* studies performed in experimental models of pulmonary hypertension¹³. Together with these data, the experiments from our study underscore the major importance of a completely intact BMPR2 signalling pathway to prevent vascular remodelling; here, this could be achieved by specific

inhibition of miR-20a, an endogenously expressed repressor of BMPR2, resulting in normalization of BMPR2 mRNA levels or overexpression of protein levels, respectively.

Taken together, we show here for the first time that the development of hypoxia-induced changes of small pulmonary arteries can be prevented by the application of antagomiR-20a, though it remains speculative whether this treatment would also reverse the once established disease. Our *in vivo* data, however, suggest that antagonisation of miR-20a inhibits vascular remodelling of pulmonary arteries in patients with pulmonary hypertension and, thus, might provide a major impact on our understanding and treatment of this disease.

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Disclosure

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Legends

Figure 1 AntagomiR-20a suppresses miR-20a expression *in vivo*

A. Study design of the *in vivo* experiments. 4 groups of 8 animals each were used; 3 groups were employed in conditions at 10% oxygen for up to 21 days and were treated with antagomiR-20a (25 mg/kg), antagomiR_MM or PBS. Injections were performed at day 0, 5 and 12. One group was used as normoxic control. B. miR-20a expression as normalized to snoRNA202 is significantly downregulated upon treatment with antagomiR-20a in lung tissue. C. Sequence of antagomiR-20a and of the mismatch control, antagomiR_MM.

Figure 2 AntagomiR-20a prevents pulmonary arterial vascular remodelling

A. Representative images of small pulmonary arteries stained against α -smooth muscle actin showing reduced hypoxia-induced remodelling of intimal and medial vessel layer upon treatment with antagomiR-20a (second from left), quantified by analysis of lung vessel occlusion (B) and thickness of vessel wall (C). Similar findings were observed for heart morphometry as shown by representative images revealing reduced right ventricular hypertrophy by treatment with antagomiR-20a (D, second from left), heart weight relative to body weight (E) and heart hypertrophy (F). Hypoxia-induced erythrocytosis was not affected significantly by treatment with antagomiR-20a (G).

Figure 3 Restoration of functional expression levels of BMPR2 by antagomiR-20a *in mice*

Treatment with antagomiR-20a increased mRNA expression of BMPR2 in lung tissue as shown by qPCR (A), immunohistochemistry (B) (antagomiR-20a treated animals shown in upper panel, antagomiR_MM in lower panel, magnification 200fold) and, (C), Western blotting. Similarly, expression of Smad 5 (D) and Id-2 (E) was increased in lung tissue by treatment with antagomiR-20a.

Figure 4 AntagomiR-20a enhances BMP-2 mediated signalling *in vitro*

Expression of Id-1 (A) and Id-2 (B) is significantly increased after stimulation with BMP-2 when transfected with antagomiR-20a (assessed at 1 and 4h). Dashed line indicates unstimulated HPASMC. C. For promoter studies, the promoter of human Id-1 was cloned containing multiple Smad

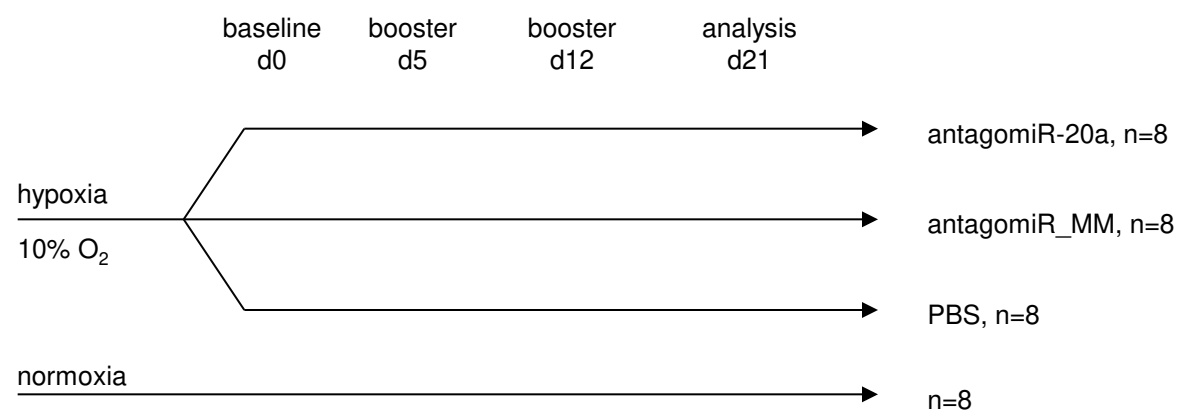
transcription factor binding sites (as described in Lopez-Rovira et al. ¹⁵). D. Reporter gene assay in HepG2 cells: Co-transfection of pGL3basic-Id-1 and antagomiR_MM or antagomiR-20a. Stimulation with BMP-2 for 4h increased response in antagomiR-20a treated cells. E. Conversely, the response to BMP-2 was reduced when miR-20a was over expressed.

Figure 5 Proliferation is reduced upon transfection with antagomiR-20a

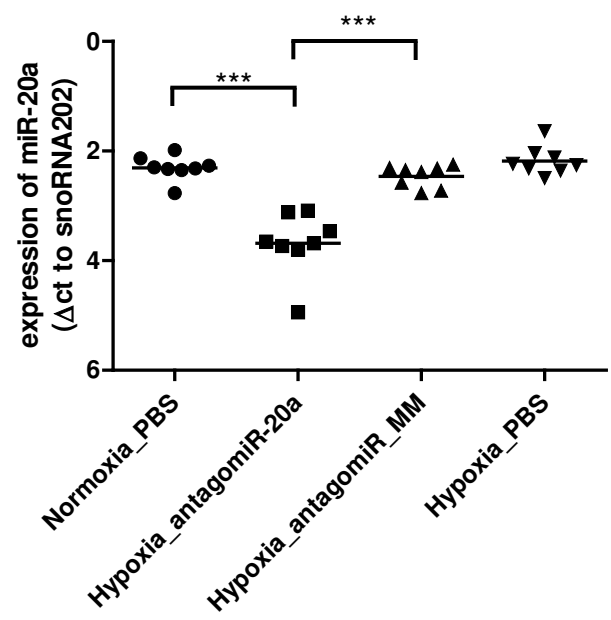
A. BrdU assay performed in HPASMC after transfection of antagomiR-20a and antagomiR_MM showed significant inhibition of cell growth as measured by absorbance at 450nm. Conversely, transfection of pre-miRs increased proliferation significantly. B. p21 was found to be up regulated in antagomiR-20a transfected HPASMC as indicated by Western Blot (at 24h of antagomiR-20a transfection). C. mRNA levels of p21 were found to be increased in HPASMC when miR-20a expression was blocked as assessed by qPCR. D. Proof of principle. miR-20a levels were found to be reduced upon transfection with antagomiR-20a.

Figure 1

A



B



C

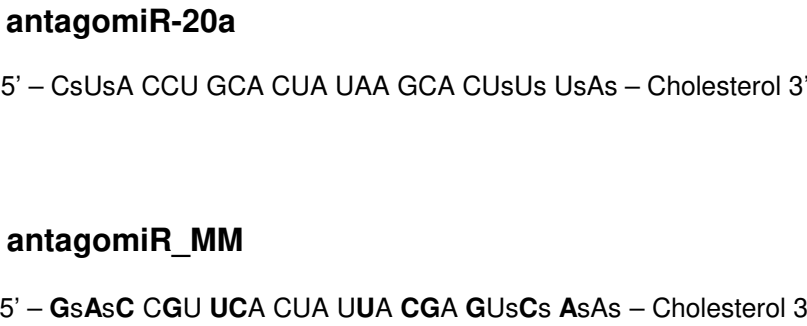


Figure 2

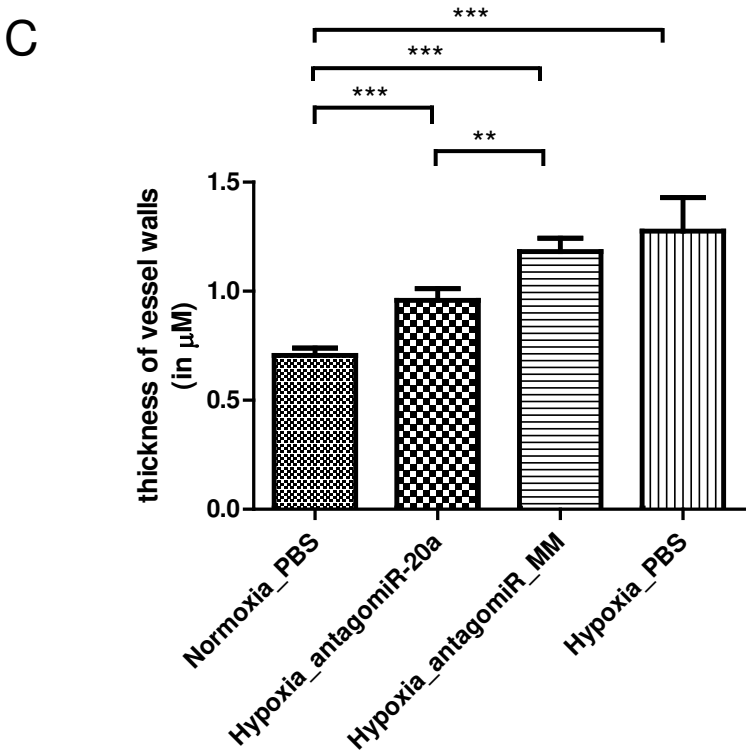
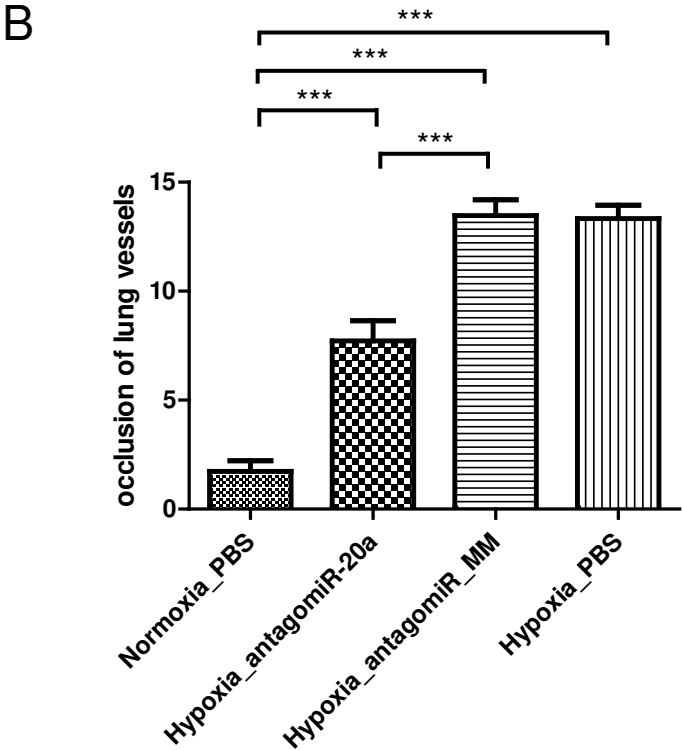
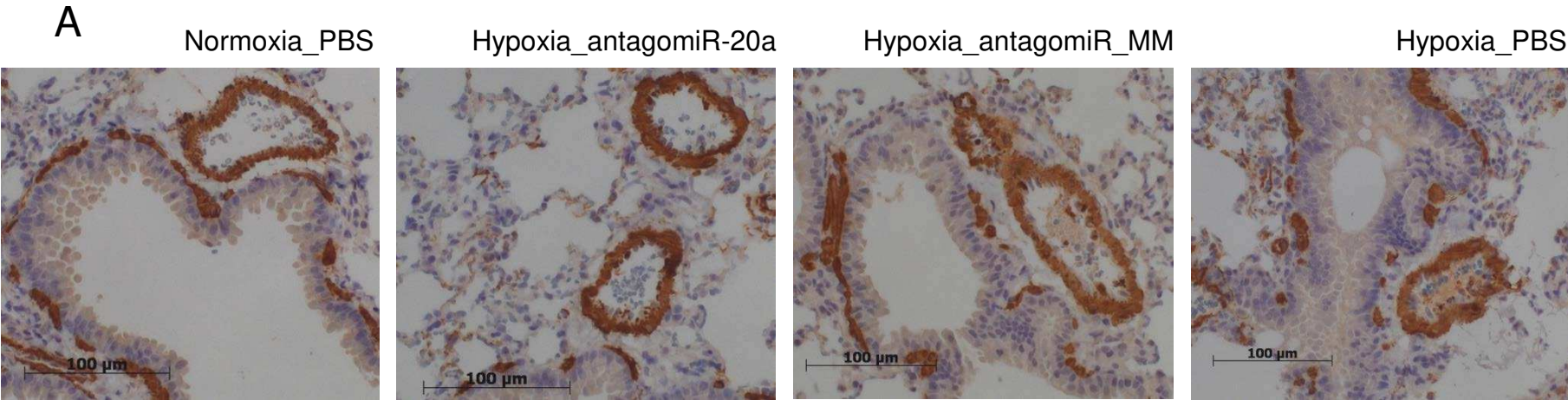


Figure 2

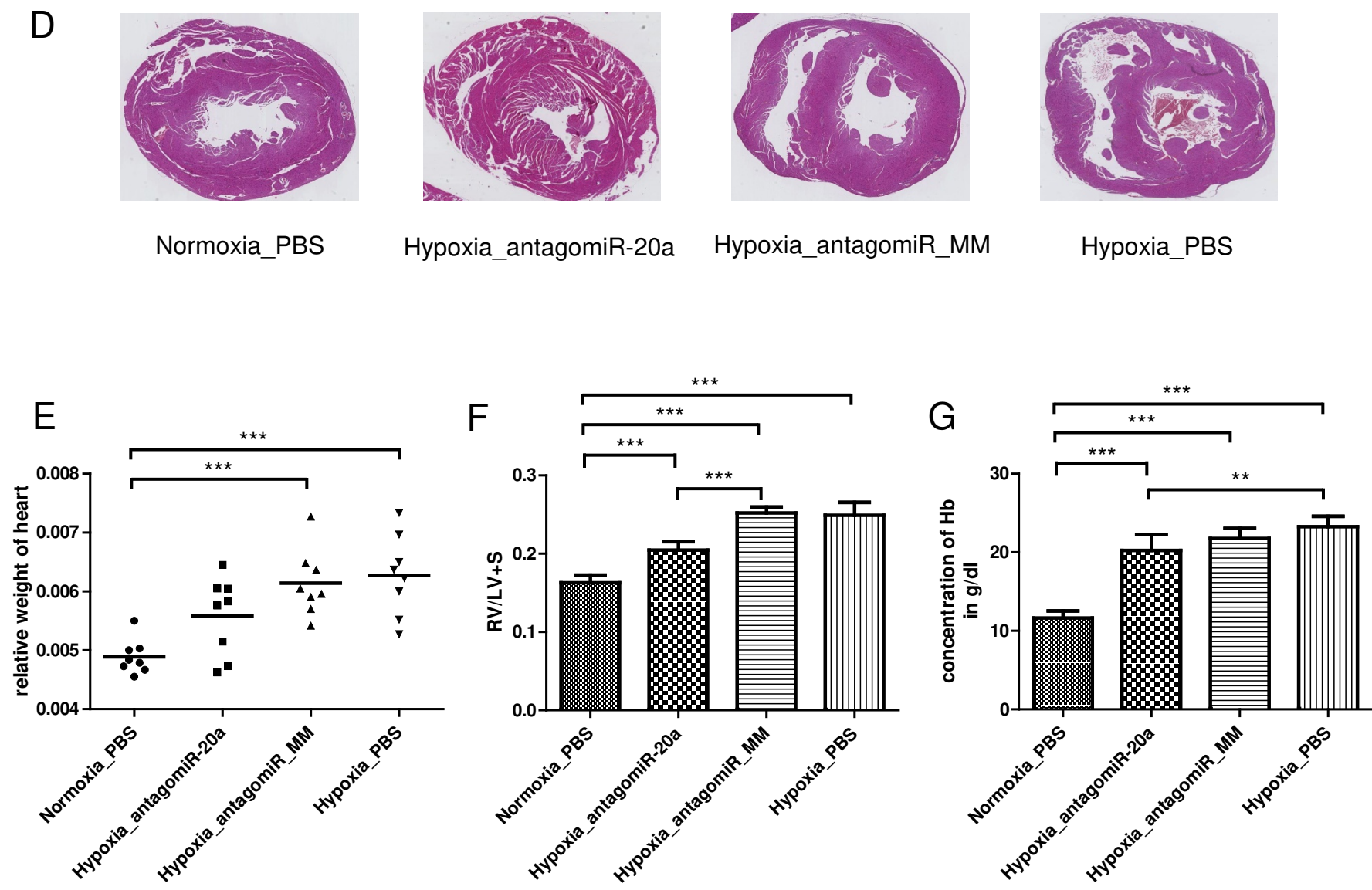
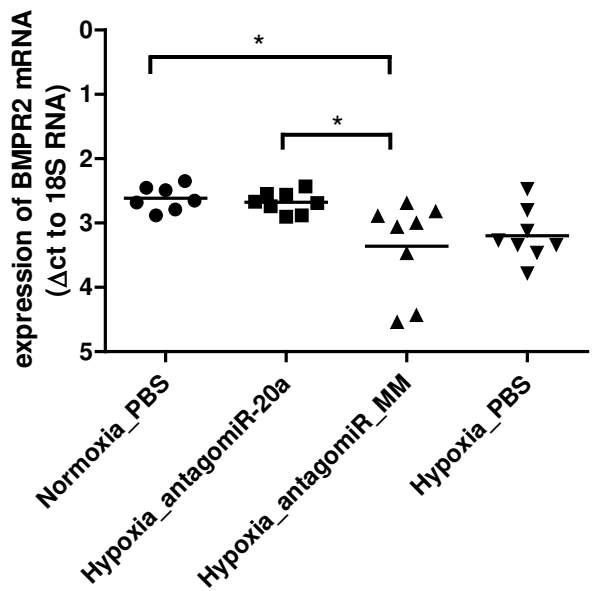
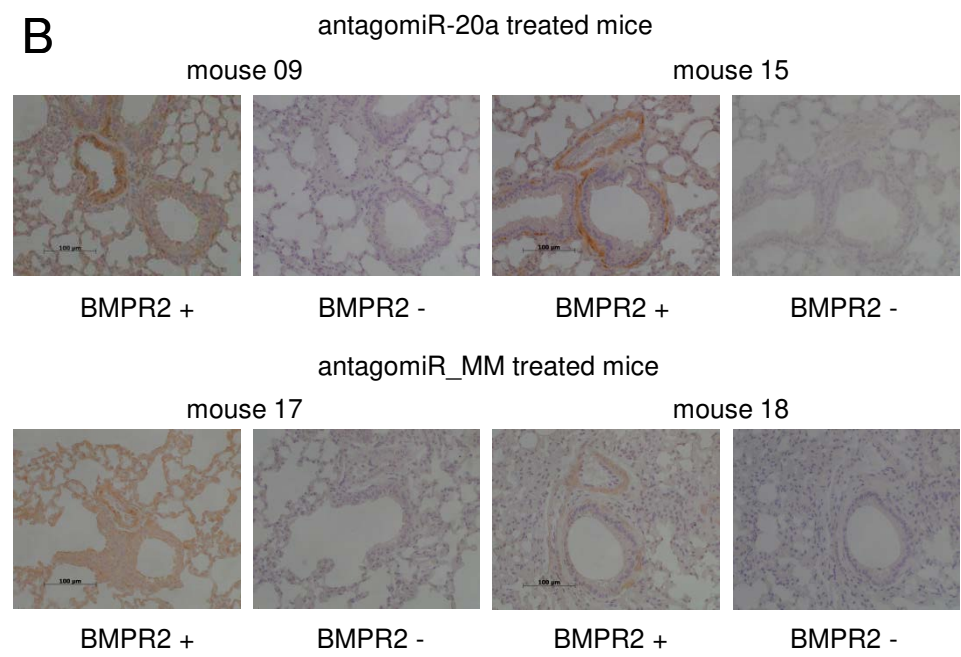


Figure 3

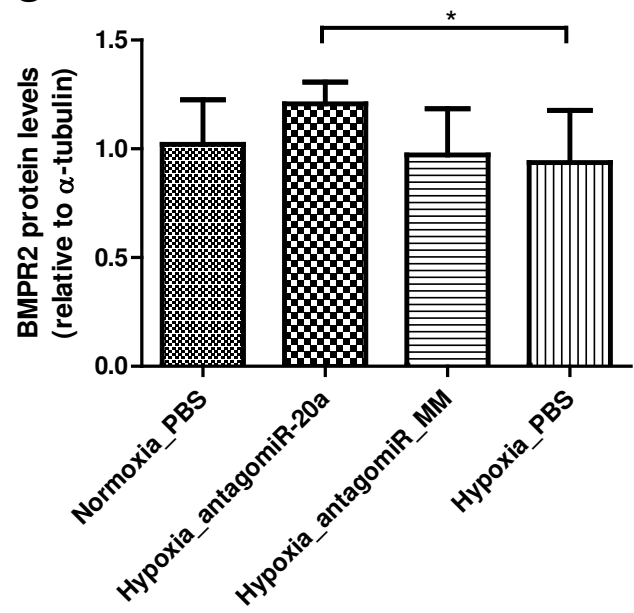
A



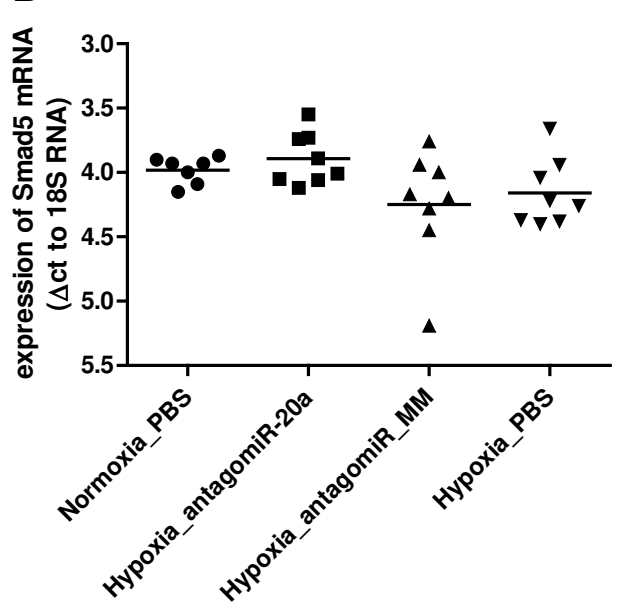
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D



E

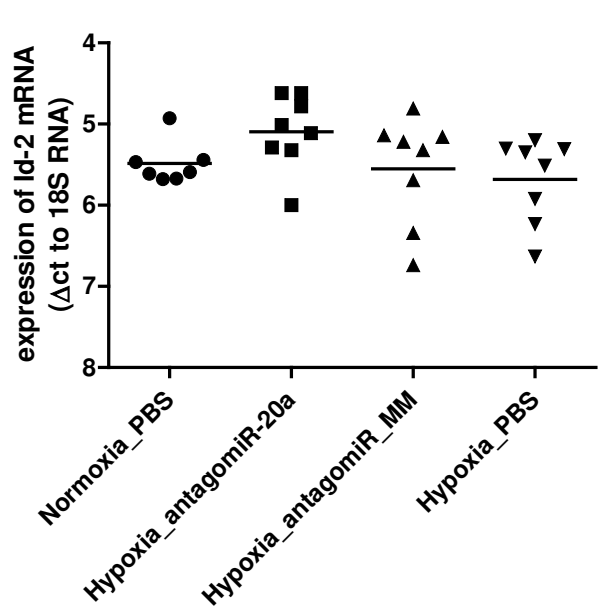
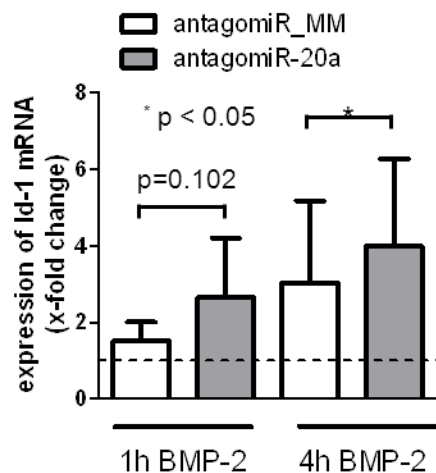
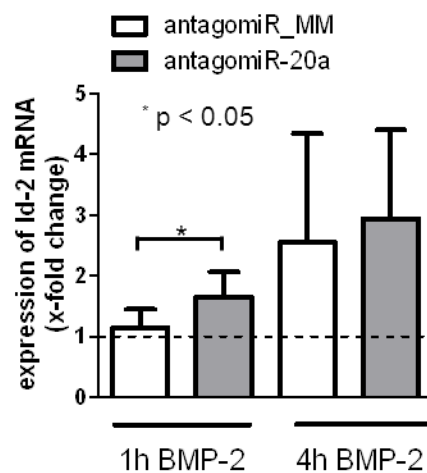


Figure 4

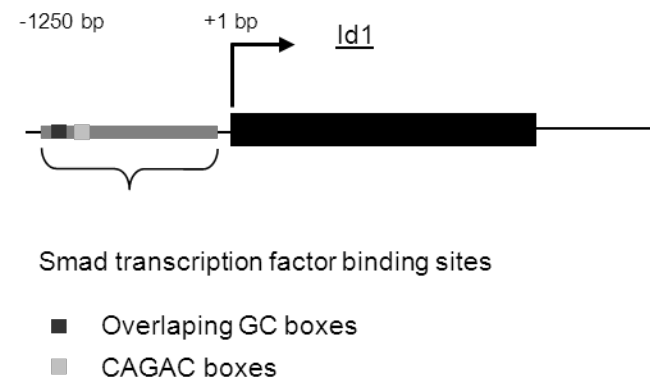
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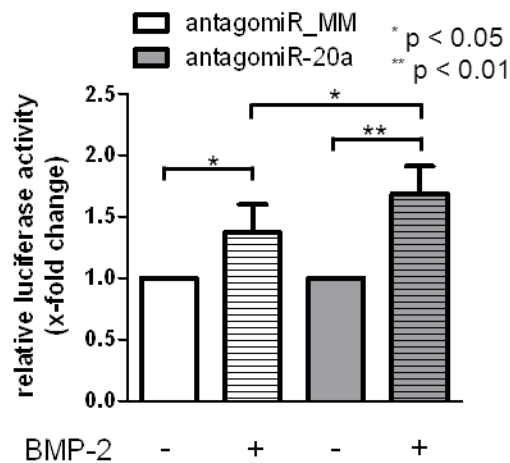
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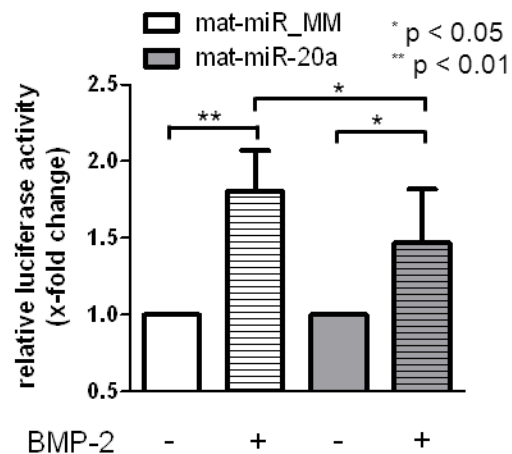
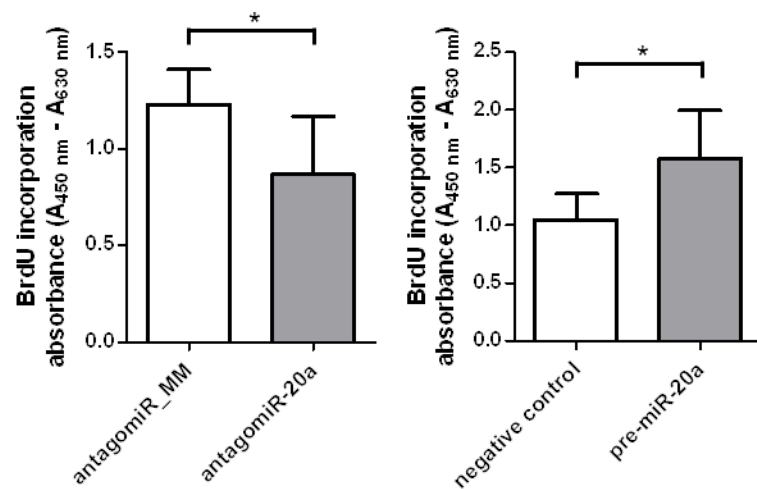
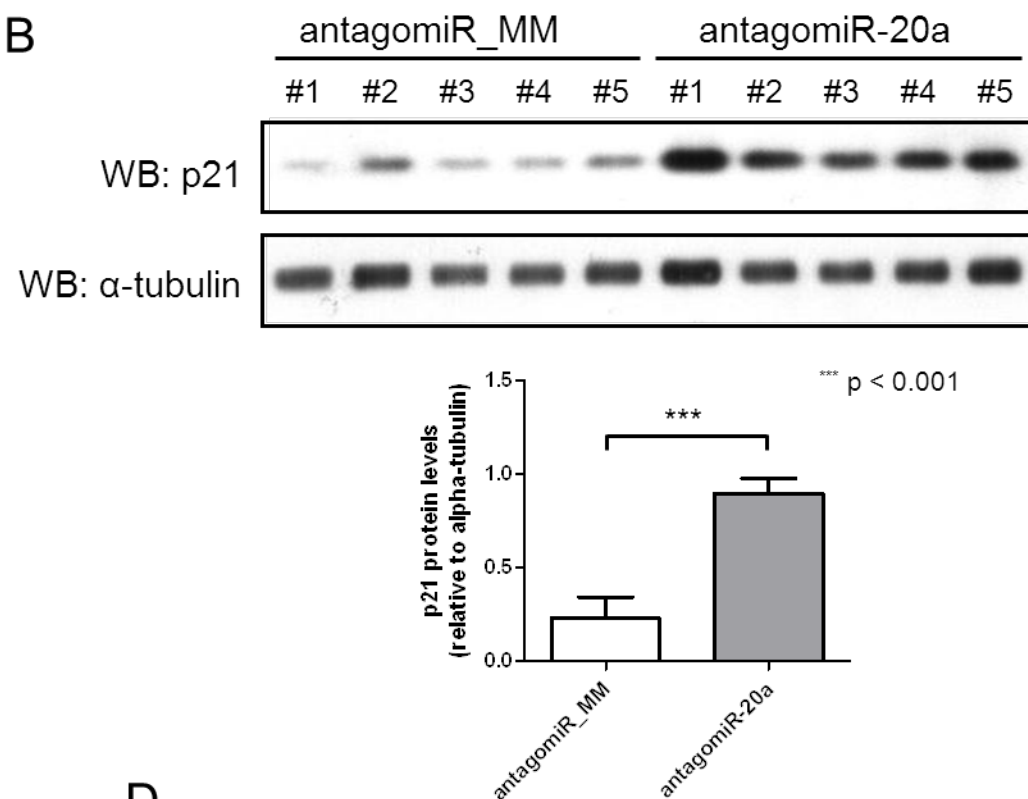


Figure 5

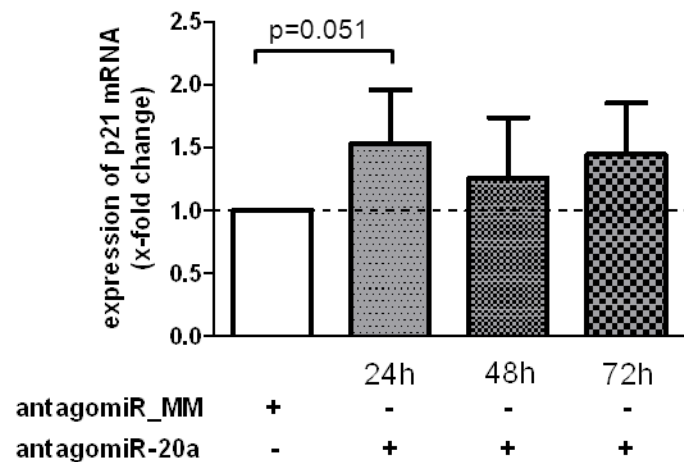
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